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Mechanism of extracellular domain-deleted dominant negative cadherins

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SUMMARY

The cadherin/catenin complex mediates Ca^{2+}-dependent cell-cell interactions that are essential for normal developmental processes. It has been proposed that sorting of cells during embryonic development is due, at least in part, to expression of different cadherin family members or to expression of differing levels of a single family member. Expression of dominant-negative cadherins has been used experimentally to decrease cell-cell interactions in whole organisms and in cultured cells. In this study, we elucidated the mechanism of action of extracellular domain-deleted dominant-negative cadherin, showing that it is not cadherin isotype-specific, and that it must be membrane-associated but the orientation within the membrane does not matter. In addition, membrane-targeted cytoplasmic domain cadherin with the catenin-binding domain deleted does not function as a dominant-negative cadherin. Expression of extracellular domain-deleted dominant-negative cadherin results in down-regulation of endogenous cadherins which presumably contributes to the non-adhesive phenotype.

Key words: E-cadherin, N-cadherin, Adherens junction, Cell adhesion, Catenin

INTRODUCTION

Cadherins comprise a large family of Ca^{2+}-dependent, homotypic cell-cell adhesion molecules that play important roles in development (reviewed by Takeichi, 1995), epithelial cell polarity (McNeill et al., 1990) and tumor progression (reviewed by Takeichi, 1993). Cadherins are single pass, type I transmembrane proteins that are localized in the adherens junction. The extracellular domain of classical cadherins consists of five 110 amino acid repeats which coordinate Ca^{2+} and are responsible for cadherin-cadherin interaction (Nagar et al., 1996). To be fully functional, the cadherin cytoplasmic domain must be linked to the actin cytoskeleton through a group of proteins termed catenins; α-catenin, β-catenin and plakoglobin (reviewed by Wheelock et al., 1996). Beta-catenin and plakoglobin interact directly with a highly conserved 30 amino acid region in the cadherin cytoplasmic domain (Stappert and Kemler, 1994) and can substitute for one another in the adherens junction in a mutually exclusive manner (Butz and Kemler, 1994; Hinck et al., 1994; Näthke et al., 1994; Sacco et al., 1995). α-Catenin interacts with the cadherin indirectly via interactions with β-catenin or plakoglobin (Sacco et al., 1995; Nieset et al., 1997; Huber et al., 1997). Thus, the adherens junction consists of a transmembrane cadherin that interacts extracellularly with an identical cadherin on an adjacent cell and intracellularly with β-catenin or plakoglobin. β-Catenin or plakoglobin interact with α-catenin which links the cadherin-catenin complex to the actin cytoskeleton via interactions with α-actinin (Knudsen et al., 1995; Nieset et al., 1997) vinculin (Hazan et al., 1997; Weiss et al., 1998; Watabe-Uchida et al., 1998) and actin filaments (Rimm et al., 1995).

Several functions of cadherins have been elucidated using dominant negative mutants. Two types of dominant negative cadherins have been described: one consisting of a cadherin molecule with a cytoplasmic deletion thus preventing association with the cytoskeleton, the second consisting of a cadherin with an extracellular deletion thus preventing cadherin self-association. For example, expression of E-cadherin with a deleted cytoplasmic domain resulted in disruption of the ectoderm in gastrulating *Xenopus* embryos (Levine et al., 1994). These authors proposed that the dominant negative effect was due to competition between the truncated cadherin and the endogenous cadherin for extracellular cadherin-cadherin interactions. The disruption was specific for E-cadherin as a similar mutation of N-cadherin did not disrupt the developing ectoderm. This is not surprising as formation of the ectoderm during gastrulation is an E-cadherin-dependent event. Neural tube formation, which is an N-cadherin-dependent event, was disrupted by a similar dominant negative N-cadherin mutation. Thus, the intracellularly deleted dominant negative cadherin disrupted developmental processes by interacting with an endogenous cadherin of the same type on an adjacent cell and, as expected, showed cadherin specificity.

Studies utilizing the second type of dominant negative cadherin, that is, with an extracellular deletion, have also been reported (Kintner, 1992; Hermiston and Gordon, 1995a,b; Fujimori and Takeichi, 1993; Amagai et al., 1995; Zhu and

Watt, 1996). Kintner (1992) described such a dominant negative N-cadherin from which he had removed most of the extracellular domain but retained the entire cytoplasmic domain. This deletion mutant was shown to be non-functional in cell aggregation experiments. When mRNA generated from this mutated cadherin was injected into *Xenopus* embryos the ectoderm began to show disruption at the stage where the embryo switches from maternal cadherin expression to embryonic cadherin expression (Choi and Gumbiner, 1989; Levi et al., 1991).

A chicken N-cadherin dominant negative cadherin has also been described and is similar to the *Xenopus* mutation described above (Fujimori and Takeichi, 1993). These authors showed that N-cadherin with an extracellular deletion (cN390Δ) disrupted cell-cell adhesion and delayed the formation of desmosomes in a mouse keratinocyte cell line (Fujimori and Takeichi, 1993; Amagai et al., 1995). In addition, an E-cadherin dominant negative mutant has been described by Zhu and Watt (1996). This deletion mutant is made up of the cytoplasmic, transmembrane, and 14 amino acids of the extracellular domains of mouse E-cadherin fused with the extracellular domain of mouse H-2K^{d}. When this cadherin deletion mutant was expressed in cultured human keratinocytes, E- and P-cadherin expression was decreased, proliferation rates were decreased and terminal differentiation was accelerated. The current study was designed to elucidate the mechanism of action of the extracellularly deleted dominant negative cadherins. We have used the cytoplasmic domain of N-cadherin and E-cadherin either targeted to the plasma membrane or soluble in the cytoplasm to show: (1) the dominant negative phenotype is not cadherin isotype-specific; (2) the dominant-negative cadherin must be membrane-associated to be active, but the orientation within the membrane is not crucial for activity; (3) the dominant negative cadherin must be able to associate with catenins in order to be active, but the mechanism does not involve merely titrating out the catenin supply; and (4) expression of the dominant negative cadherin results in decreased expression of endogenous cadherins by increasing the turnover rate of the endogenous cadherin.

MATERIALS AND METHODS

Cell culture

The human epidermoid carcinoma cell line A431 was obtained from ATCC (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Hyclone Laboratories, Logan, UT).

Antibodies and reagents

Unless otherwise stated, all reagents were from Sigma Chemical Co. (St Louis, MO). Rabbit polyclonal antibodies (Jelly) against human E-cadherin extracellular domain (Wheelock et al., 1987) and mouse monoclonal antibodies against α-catenin (1G5; Johnson et al., 1993), plakoglobin (15F11; Sacco et al., 1995), E-cadherin (HECD1; a kind gift from Dr Masatoshi Takeichi, Kyoto University, Kyoto, Japan) and N-cadherin (13A9; Knudsen et al., 1995; Sacco et al., 1995) have been previously described. The mouse monoclonal antibodies against β-catenin (6E3) and the cytoplasmic domain of E-cadherin (4A2) were made as previously described (Johnson et al., 1993). The mouse monoclonal antibody against erbB2 was purchased from Transduction Laboratories (Lexington, KY). The rabbit polyclonal anti-α-catenin was purchased from Sigma Chemical Co.

Molecular constructs and transfections

DNA encoding the complete cytoplasmic domain of N-cadherin or E-cadherin was generated using PCR and subcloned into pSPUTK, a vector that adds the 5′ untranslated region (UTR) of globin (Falcone and Andrews, 1991). The cytoplasmic domain of N-cadherin or E-cadherin and the globin 5′ UTR were ligated into the pLK-pac expression vector (Islam et al., 1996); these clones are referred to as N-cad-CD or E-cad-CD (Fig. 1A). To target cadherin intracellular domains to the plasma membrane, N-cad-M and E-cad-M (Fig. 1A) were constructed by ligating the cytoplasmic domain of the cadherin, the nucleotides encoding the amino-terminal plasma membrane targeting sequence (MGSSKSKPKDPSQR) from the vector M (Aronheim et al., 1994) and the 5′ UTR of globin into pLK-pac. N-cad-F (Fig. 1C) was constructed by ligating the cytoplasmic domain of N-cadherin, the nucleotides encoding the carboxyl-terminal plasma membrane targeting sequence (KLNPPDQSGPGCMSCKCVLM) from the vector F (Aronheim et al., 1994) and the 5′ UTR of globin into pLK-pac. Vectors M and F were kind gifts from Dr Ami Aronheim (University of California, San Diego, School of Medicine). E-cad-myc was constructed by adding a 2×-myc epitope to the last codon. All constructions were sequenced and shown to encode the correct amino acid sequences.

Cells were transfected using a calcium phosphate kit (Stratagene, La Jolla, CA). In every case, stable clones were selected by growth in puromycin (1 μg/ml). Expression of the MMTV driven transgene was induced with dexamethasone (10^{-7} M) for 24 hours and stably transfected clones were shown to express the transgene by immunoblot analysis.

Microscopy

Cells were grown in DMEM, 10% FCS with dexamethasone (10^{-7} M) on glass coverslips until almost confluent, fixed in Histochoice (Amresco, Solon, OH) permeabilized in 100% cold methanol for 4 minutes at −20°C. Coverslips were blocked for 1 hour in PBS containing 10% goat serum and stained with primary antibodies for 1 hour followed by FITC or rhodamine-conjugated anti-IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Fluorescence was detected with a Zeiss Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY) and photographed using T-Max 3200 film.

Protein assays

Quantification of cell extracts was done using the Bio-Rad Protein Assay reagent (Richmond, CA).

Immunoprecipitations

Cells were grown to confluence and either extracted as described (Lewis et al., 1994) or metabolically labeled with [^{35}S]methionine/cysteine. Monolayers of cells were washed three times with PBS at room temperature, extracted at 4°C with 2 ml/75 cm^2 flask of TNE (10 mM Tris acetate, pH 8.0, 0.5% NP-40, 1 mM EDTA) saturated with phenylmethylsulfonylfluoride (PMSF). The cells were removed from the flask by scraping followed by vigorous agitation for 10 minutes on ice. Insoluble material was removed by centrifugation at 15,000 ***g*** for 15 minutes. Primary antibodies were added to unlabeled or ^{35}S-labeled extracts and mixed at 4°C for 30 minutes. Anti-mouse IgG-Sepharose (ICN, Costa Mesa, CA) was added and mixing continued for an additional 30 minutes. The Sepharose-bound immune complexes were washed five times with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% SDS and 0.5% deoxycholic acid for ^{35}S-labeled cell extracts or with 10 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.5% Tween (Fisher, Fair Lawn, NJ) for unlabeled cell extracts. Pellets of Sepharose-bound immune complexes were boiled in Laemmli sample buffer (Laemmli, 1970) and resolved on 7% SDS-PAGE as described (Lewis et al., 1994). For gels with labeled

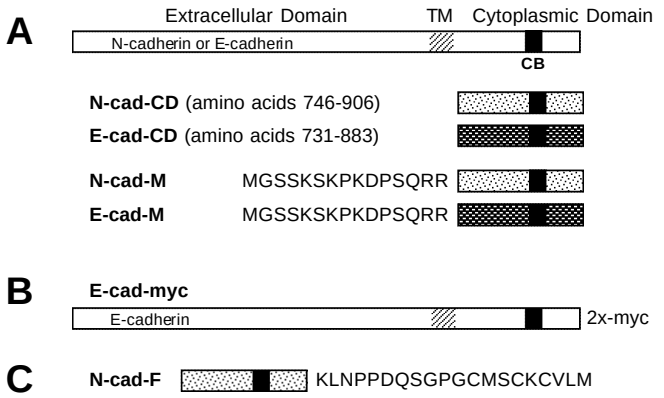


Fig. 1. Molecular constructions. (A) The cytoplasmic domains of N-cadherin (amino acids 746-906) or E-cadherin (amino acids 731-883) were generated with PCR (N-cad-CD or E-cad-CD, respectively). The amino-terminal plasma membrane targeting sequence from src was added to the cytoplasmic domain of N-cadherin or E-cadherin (N-cad-M or E-cad-M, respectively). The catenin-binding site (CB) and transmembrane domain (TM) are pointed out. (B) E-cad-myc is full length human E-cadherin with a 2×-myc tag added to the carboxyl terminus. (C) N-cad-F is the cytoplasmic domain of N-cadherin with the addition of the carboxyl-terminal plasma membrane targeting sequence from ras.

extracts ^{14}[C]-labeled molecular mass markers (Gibco) included myosin (205 kDa), phosphorylase b (97 kDa), BSA (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa). For gels with unlabeled extracts molecular mass markers included the above plus β-galactosidase (116 kDa).

Gels containing labeled extracts were transferred electrophoretically to nitrocellulose and autoradiographed with Kodak BioMax XR film at −70°C using Kodak BioMax Transcreen LE intensifying screens. Gels containing unlabeled extracts were transferred to nitrocellulose and immunoblotted as described (Wheelock et al., 1987). Bands were scanned using a PDI flatbed scanner and quantified with Quantity One, version 3.0 (PDI, Huntington Station, NY).

Metabolic labeling of cells

Cells (7×10^5) were plated in 25 cm^2 flasks and grown for 12 hours in DMEM supplemented with 10% FCS. Culture medium was replaced with cysteine/methionine-deficient DME supplemented with 1% dialyzed FCS for 4 hours. The cells were labeled with [^{35}S]methionine/cysteine] (Translabel, ICN, Costa Mesa, CA) for 30 minutes then chased with DMEM supplemented with 10% FCS for 4, 8, 12 or 16 hours and extracted with 0.5 ml TNE.

Cell fractionation

Confluent 225 cm^2 flasks of cells were washed with PBS and scraped into 5 ml of TE (10 mM Tris acetate (pH 8.0), 1 mM EDTA) saturated with PMSF at 0°C and Dounce homogenized until the majority of the cells were broken but nuclei remained intact as determined by phase microscopy. The extract was centrifuged at 15,000 ***g*** for 15 minutes. The supernatant was designated as the aqueous soluble fraction. The pellet was washed once with TE, extracted with 5 ml 0.5% NP-40 in TE and centrifuged again at 15,000 ***g*** for 15 minutes. The supernatant from this fraction was designated the NP-40 soluble fraction.

Ribonuclease protection assay

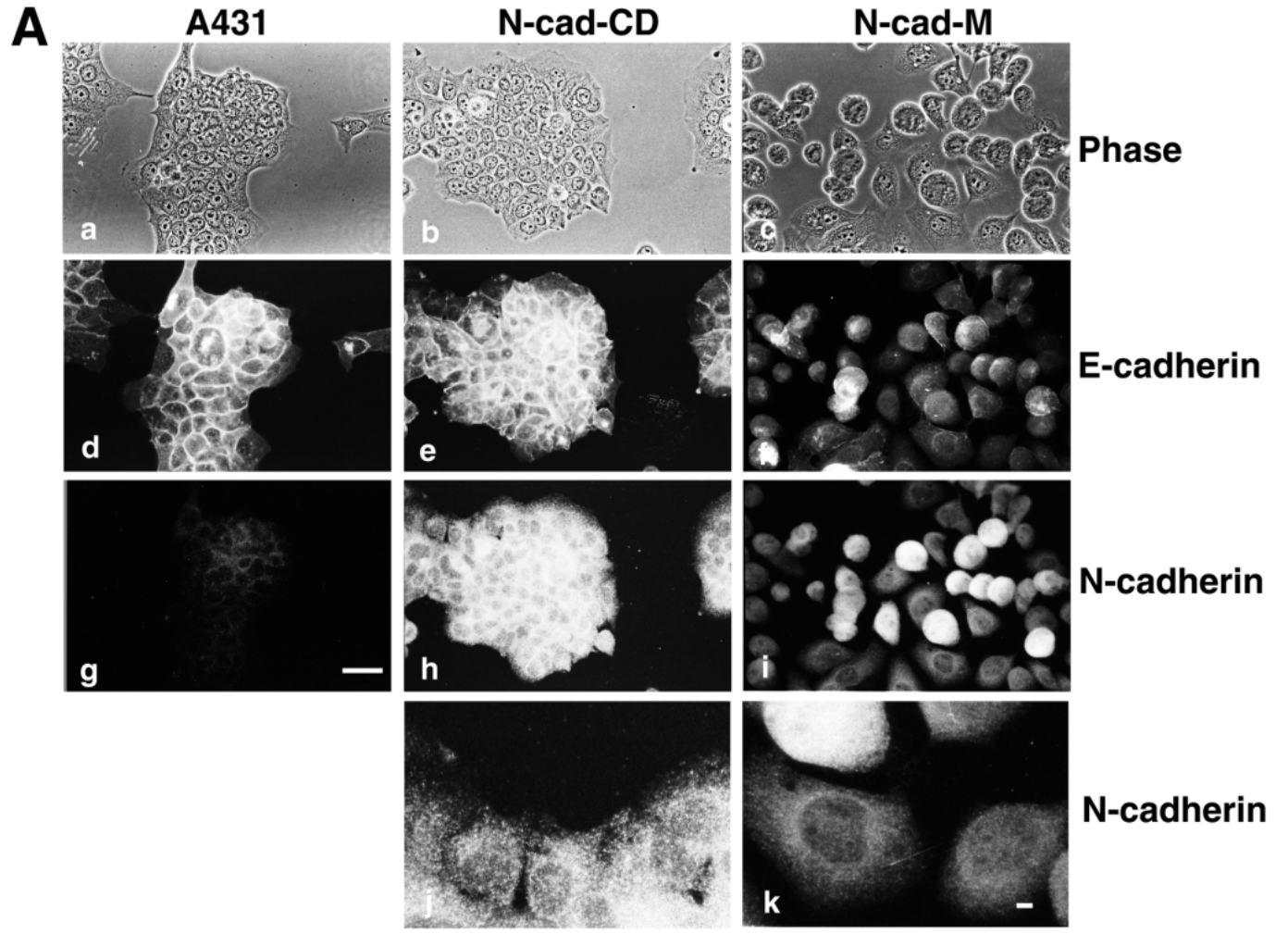
Total RNA was extracted from cells using RNA Isolator (Genosys Biotechnologies, Inc., Woodlands, TX). Probe sets were generated by cloning PCR products corresponding to nucleotides (nt) 1682-2042 for E-cadherin (GenBank Z13009), nt 1463-1782 for P-cadherin (GenBank X63629), nt 279-506 for α-catenin (GenBank D13866), nt 348-602 for β-catenin (GenBank X87838), or nt 221-442 for plakoglobin (GenBank M23410) and ligated into pGEM4 (Promega, Madison, WI). The probe for L32, a kind gift from Dr Rosemary Rochford (University of Michigan School of Public Health, Ann Arbor, MI) has been described (Rochford et al., 1993). Cadherin and catenin mRNAs were quantified using an RNAse protection assay (Hobbs et al., 1993). To synthesize radiolabeled anti-sense probe sets, the final reaction (10 μl) contained 120 μCi [α-^{32}P]UTP (3000 Ci/mmol, Amersham, Arlington Heights, IL); UTP (73 pmol); ATP, CTP and GTP (2.8 nmol each); DTT (100 nmol); transcription buffer; RNAsin (12 U); T7 polymerase (10 U) (all from Ambion, Austin TX) and equimolar amounts of each linearized probe (60 ng DNA total). After 1 hour incubation at 37°C, the reaction was terminated by adding DNAse (2 U, Ambion) and incubating 30 minutes at 37°C. The labeled probes were purified by phenol/chloroform and chloroform extraction followed by ethanol precipitation. The probes were dissolved (3×10^5 cpm/μl) in hybridization buffer (80% formamide, 0.4 M NaCl, 1 mM EDTA, 40 mM Pipes, pH 6.7) and 2 μl were added to tubes containing 5 μg total RNA in 8 μl of hybridization buffer, heated to 90°C and incubated at 56°C for 16 hours. Single stranded RNA was digested by treating each sample with RNAse T1 (1500 U, Gibco BRL, Gaithersburg, MD, 10 mM Tris, 300 mM NaCl, 5 mM EDTA, pH 7.5) for 30 minutes at 30°C, followed by addition of 18 μl of proteinase K (0.5 mg/ml, Boehringer Mannheim Corp., Indianapolis, IN, SDS, 3.5%, yeast tRNA, 0.13 μg/μl) and incubation for 30 minutes at 37°C. The protected probes were extracted, precipitated, dissolved in loading buffer (80% formamide, 1 mM EDTA, 50 mM Tris-borate, pH 8.3), separated on a 5% acrylamide/8 M urea gel and autoradiographed at −80°C with intensifying screens.

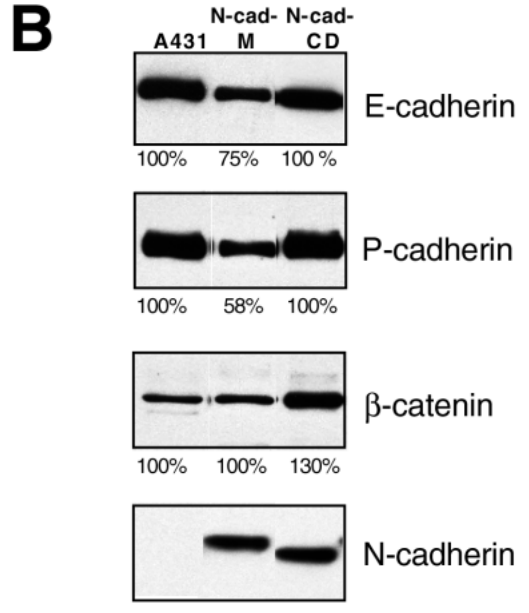
RESULTS

Localization at the plasma membrane is essential for the dominant negative phenotype

To construct a plasma membrane-targeted cadherin cytoplasmic domain without the influence of the transmembrane domain or residual extracellular amino acids the myristoylation signal (MGSSKSKPKDPSQR) derived from src was added to cadherin intracellular domains (see Fig. 1A). During translation of src the N-terminal methionine is removed leaving glycine at the N terminus. Glycine is then modified by covalent attachment of myristic acid through an amide bond (Towler et al., 1988). Myristoylation is not sufficient for src to be directed to the plasma membrane; the basic nature of the amino acid sequence is also important for proper targeting and for stable interaction with the membrane (Gordon et al., 1991; Resh, 1994).

A431 cells stably transfected with membrane-targeted dominant negative N-cadherin (N-cad-M) underwent a dramatic morphological change when compared to parental A431 cells. In contrast, A431 cells stably transfected with the soluble cytoplasmic domain of N-cadherin (N-cad-CD) were indistinguishable from the parental cells (Fig. 2A, a-c). Parental cells and A431-N-cad-CD cells displayed a typical epithelial morphology and grew in compact colonies, whereas A431-N-cad-M cells were more rounded and tended to grow as single cells. Immunofluorescence microscopy revealed that A431-N-cad-CD cells, like the parental A431 cells, had distinct cell-cell border staining for endogenous E-cadherin, whereas A431-N-cad-M cells had drastically reduced and disorganized E-cadherin staining (Fig. 2A, d-f).





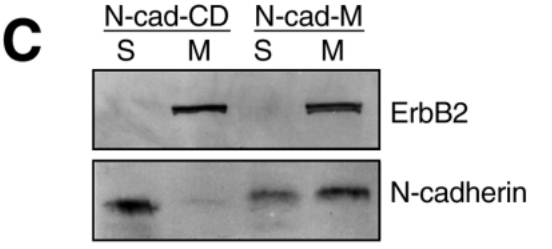


Fig. 2. The cytoplasmic domain of N-cadherin disrupted the epithelial morphology and decreased the level of E-cadherin staining when targeted to the plasma membrane in A431 cells. (A) A431 cells (a,d,g), A431-N-cad-CD cells (b,e,h,j) or A431-N-cad-M cells (c,f,i,k) were processed for co-immunofluorescence localization of E-cadherin using the rabbit polyclonal antiserum, Jelly (d-f) and N-cadherin using the mouse monoclonal antibody 13A9 (g-k). Phase contrast microscopy was used to compare the morphologies of the three cell lines (a-c). Note the altered morphology and decreased E-cadherin staining in N-cad-M cells, but not N-cad-CD cells. (j and k) Higher magnification pictures of the cells in h and i, respectively, to further point out the diffuse distribution of N-cad-CD and N-cad-M. Bars, 10 μM. (B) Confluent monolayers of cells were extracted and equal amounts of protein resolved by 7% SDS-PAGE and immunoblotted with antibodies against E-cadherin (HECD-1), P-cadherin (6A9), β-catenin (6E3) or) N-cadherin (13A9). (C) Cell extracts were separated into soluble or membrane associated fractions, resolved by 10% SDS-PAGE and immunoblotted with anti-N-cadherin (13A9) or anti-erbB2.

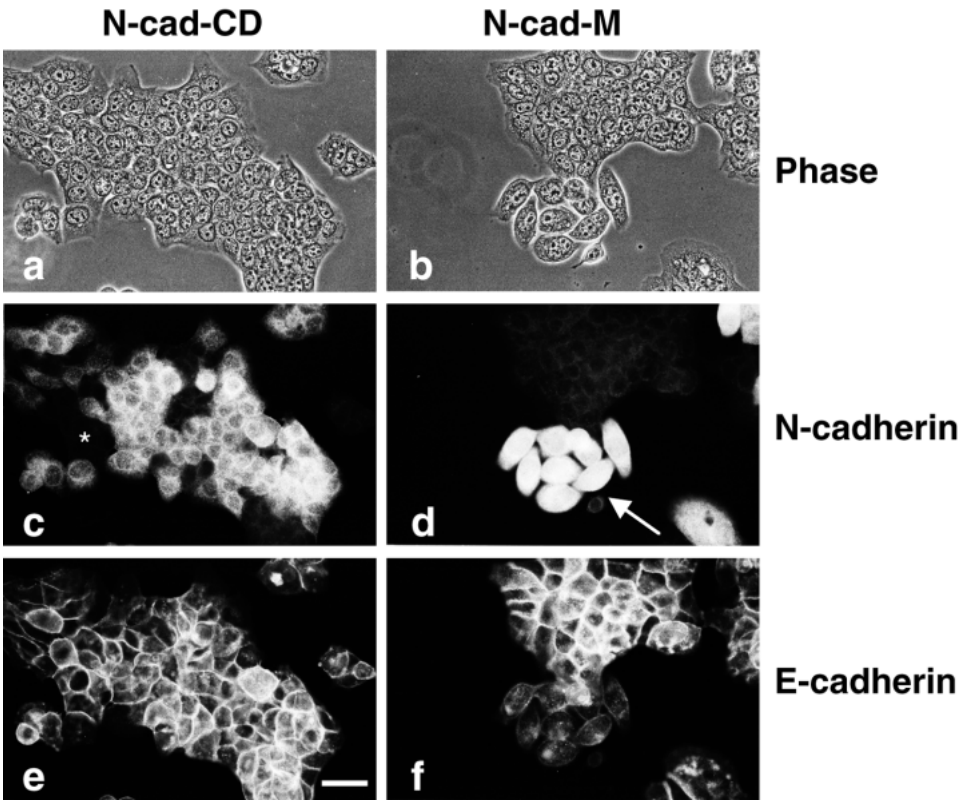


Fig. 3. A431 cells segregate from A431-N-cad-M cells, but not A431-N-cad-CD cells. Equal numbers of A431 cells and A431-N-cad-CD cells (a,c,e) or A431 cells and A431-N-cad-M cells (b,d,f) were co-cultured and processed for double-label immunofluorescence with antibodies against E-cadherin (Jelly) and N-cadherin (13A9). Phase contrast microscopy was used to examine the morphology of the mixed populations of cells (a and b). N-cad-CD and N-cad-M cells were distinguished from A431 cells by staining for N-cadherin (c and d). The asterisk in c indicates a group of non-transfected A431 cells that are mixed with the N-cad-CD cells and are indistinguishable by phase microscopy. The arrow in d points out a group of N-cad-M cells that are segregated from the surrounding non-transfected A431 cells and are clearly distinguishable by phase microscopy. Note that E-cadherin staining is continuous between A431 cells and N-cad-CD cells (e), but is not continuous between A431 cells and N-cad-M cells (f). Bar, 10 μM.

To further evaluate changes in cadherin expression when A431 cells were transfected with the cadherin cytoplasmic domain constructs, we performed quantitative immunoblot analysis (Fig. 2B). A431-N-cad-M cells showed a decrease in endogenous E-cadherin and P-cadherin protein when compared to parental A431 cells, whereas A431-N-cad-CD cells expressed levels of E- and P-cadherin comparable to the parental cell line. The level of β-catenin was increased in A431-N-cad-CD cells as compared to the parental cells (Fig. 2B), which paralleled the increased total cadherin cytoplasmic domain available for catenin-binding. Identical protein samples were immunoblotted with antibodies against N-cadherin to assess the levels of expression of the transfected N-cadherin cytoplasmic domains (Fig. 2B).

Immunofluorescence microscopy using antibodies against N-cadherin revealed diffuse staining in both N-cad-CD and N-cad-M transfected A431 cells (see Fig. 2A, h-k). Since immunofluorescence cannot distinguish between diffuse membrane localization and diffuse cytosolic localization, transfected cells were fractionated into soluble and membrane associated fractions as described in Materials and Methods. Fig. 2C shows that N-cad-CD was in the soluble fraction while a significant portion of the N-cad-M was in the membrane associated fraction, indicating that N-cad-M was effectively targeted to the plasma membrane whereas N-cad-CD was soluble within the cytosol. ErbB2 was used as a membrane protein marker for these studies.

One function of cadherins is to mediate the segregation of different cell types; i.e. cells expressing the same cadherin(s) interact with one another and cells expressing different cadherins do not (reviewed by Takeichi, 1995). In addition, cells expressing different levels of the same cadherin have been shown to segregate from one another (Steinberg and Takeichi, 1994). To determine if expression of N-cad-M or N-cad-CD influenced the ability of the transfected A431 cells to associate with parental A431 cells, we prepared co-cultures of parental cells mixed with stably transfected cells. Morphological analysis of the co-cultures showed that A431 cells and cells stably transfected with N-cad-CD formed mixed colonies and were indistinguishable from one another, whereas cells stably transfected with N-cad-M did not mix with A431 cells and were readily distinguished from the parent cell line (Fig. 3, a and b). Co-immunolocalization of the transfected N-cadherin fragment with endogenous E-cadherin confirmed the identity of transfected cells and parental A431 cells. The N-cad-CD transfected cells mixed with the A431 cells and it was impossible to distinguish the two cell lines from one another by staining for E-cadherin (Fig. 3e). As expected, staining for N-cadherin readily distinguished between the two populations of cells; a group of N-cadherin-negative parental cells is pointed out by an asterisk in c. N-cad-M transfected cells segregated from the parental cells and were readily identified by the presence of N-cadherin and reduced expression of E-cadherin. N-cadherin-positive cells are pointed out by an arrow in d; note that these cells show decreased staining for E-cadherin (f).

Expression of the cytoplasmic domain of E-cadherin produced a dominant negative effect similar to that of N-cadherin

Previous studies from our laboratory suggested that N-cadherin generates a specific signal in epithelial cells that differs from signals generated by E-cadherin. Oral squamous epithelial cells that express N-cadherin are more invasive than N-cadherin-

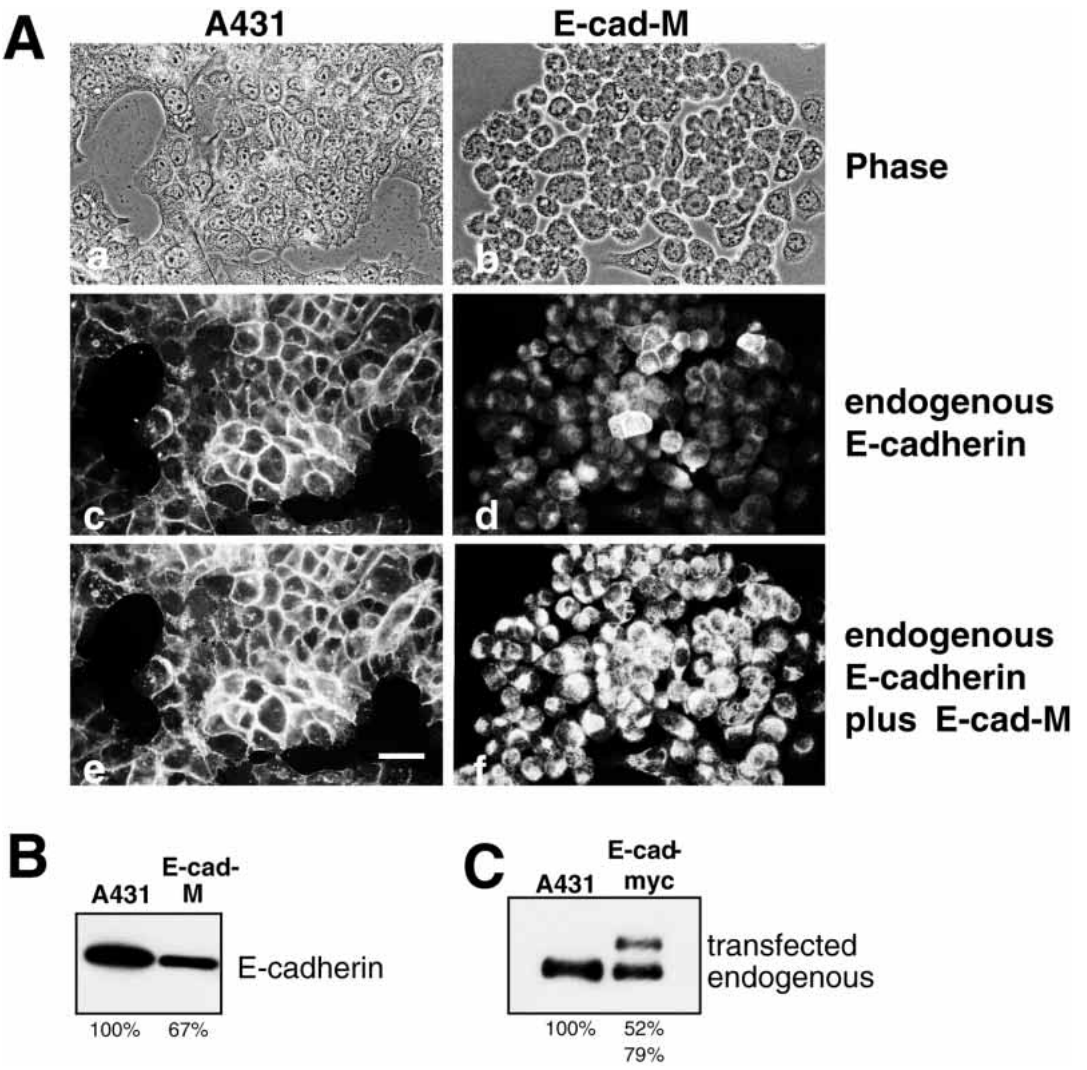


Fig. 4. A431-E-cad-M cells display a phenotype similar to that of A431-N-cad-M cells. (A) A431 or A431-E-cad-M (E-cad-M) cells were processed for co-immunofluorescence with antibodies against the extracellular domain of E-cadherin (Jelly) and antibodies against the cytoplasmic domain of E-cadherin (4A2). Phase contrast microscopy was used to compare cellular morphologies (a and b) Endogenous E-cadherin staining was detected with the extracellular domain antibody (c and d). Endogenous plus transfected E-cad-M were detected using the cytoplasmic domain antibody (e and f). Note the decrease in endogenous E-cadherin and loss of epithelial morphology in A431-E-cad-M cells. Bar, 10 μM. (B) Confluent monolayers of cells were extracted and equal amounts of protein resolved by SDS-PAGE and immunoblotted with antibodies against the extracellular domain of E-cadherin (HECD-1). Note the decrease in endogenous E-cadherin. (C) Confluent monolayers of A431 cells or A431 cells transfected with myc-tagged E-cadherin (E-cad-myc) were extracted and equal amounts of protein resolved by SDS-PAGE and immunoblotted with antibodies against the extracellular domain of E-cadherin (HECD-1). Note the decrease in endogenous E-cadherin with a concomitant increase in transfected, myc tagged E-cadherin.

negative cells. In addition, N-cadherin expressing cells have decreased levels of E-cadherin and show a fibroblastic morphology (Islam et al., 1996). These observations led us to ask whether the loss of adhesion observed when A431 cells express the membrane associated dominant negative N-cadherin is specific to N-cadherin. To address this question, we prepared E-cad-CD and E-cad M constructs (Fig. 1A) and stably transfected them into A431 cells. Phase contrast microscopy, immunofluorescence localization of endogenous E-cadherin (Fig. 4A), immunoblot analysis of E-cadherin levels (Fig. 4B) and cell fractionation (data not shown) experiments all gave results similar to those seen with the N-cadherin constructs. Cells expressing the membrane associated E-cad-M showed a disrupted epithelial morphology (Fig. 4A, b) and a diminished level of endogenous E-cadherin (Fig. 4A, d) whereas cells expressing E-cad-CD were indistinguishable from the parental A431 cell line (data not shown). In these studies, endogenous E-cadherin was detected by staining cells with a rabbit polyclonal antibody against the extracellular domain of the cadherin while both endogenous and transfected cadherin were detected by staining with a monoclonal antibody against the cytoplasmic domain. These data indicate that the dominant negative phenotype is not specific for any particular cadherin but rather is likely to be a general phenomenon of classical cadherins.

The above experiments indicated that the cytoplasmic

domain of N-cadherin and E-cadherin were both efficient at down-regulating endogenous E-cadherin. In addition, our previous work showed that transfecting full-length N-cadherin into squamous epithelial cells resulted in down-regulation of endogenous E-cadherin (Islam et al., 1996). Thus, we wanted to determine if transfecting a functional E-cadherin into A431 cells would decrease the level of endogenous E-cadherin. Therefore, we constructed full length E-cadherin with a myc tag added to the carboxyl terminus (E-cad-myc, Fig. 1B) to distinguish endogenous E-cadherin from exogenous, transfected E-cadherin. E-cad-myc was stably transfected into A431 cells (A431-E-cad-myc cells) and was found to be localized at the plasma membrane and to bind catenins (data not shown). As predicted, there was no difference in morphology between A431 and A431-E-cad-myc cells since the transfected E-cad-myc was functional in adhesion (data not shown). However, the level of endogenous E-cadherin in A431-E-cad-myc cells was decreased to 79% of the parental level (Fig. 4C), which is similar to what we saw in cells expressing the dominant negative cadherins. Since exogenous expression of either the functional cadherin (E-cad-myc) or non-functional cadherin (N-cad-M and E-cad-M) decreased endogenous E-cadherin, we speculated that the effect on endogenous E-cadherin was due to increased expression of the cadherin cytoplasmic domain.

Association with catenins is necessary, but not sufficient, for the decrease in E-cadherin expression

Hinck et al. (1994) reported that, in MDCK cells, the cadherin-catenin complex is not completely assembled until it reaches the plasma membrane. These authors showed that β-catenin attached to E-cadherin as the cadherin traveled through the endoplasmic reticulum, but α-catenin joined the complex only after β-catenin/cadherin was at the cell surface. Thus, it seemed possible that the non-membrane bound, cytoplasmic domain of cadherin did not function as a dominant negative because it could not bind catenins. To test this idea, we immunoprecipitated N-cad-M and N-cad-CD from cells expressing these proteins and examined the immunoprecipitation reactions for the presence of α-catenin, β-catenin and plakoglobin (Fig. 5A, lanes 2 and 3). Antibodies to N-cadherin co-immunoprecipitated α-catenin, β-catenin and plakoglobin from both A431-N-cad-CD cells and A431-N-cad-M cells, but not from parental A431 cells which do not express endogenous N-cadherin. Interestingly, N-cad-CD appeared to compete effectively with endogenous cadherins for α-catenin, β-catenin and plakoglobin even though it was not at the plasma membrane and was synthesized on free ribosomes. Competition with the endogenous cadherin for a limited supply of catenins has been suggested as the mechanism of action of dominant negative cadherins (Kintner, 1992). To test this hypothesis, we immunoprecipitated endogenous E-cadherin from A431, A431-N-cad-CD and A431-N-cad-M cells and probed for α-catenin, β-catenin and plakoglobin (Fig. 5A, lanes 4-6). Alpha-catenin, β-catenin and plakoglobin co-immunoprecipitated with the endogenous E-cadherin suggesting that competition for catenins is not sufficient to produce the dominant negative phenotype. It appears that plakoglobin association with both N-cad-M and E-cadherin is decreased in cells transfected with N-cad-M when compared with parental A431 cells or cells stably transfected with the

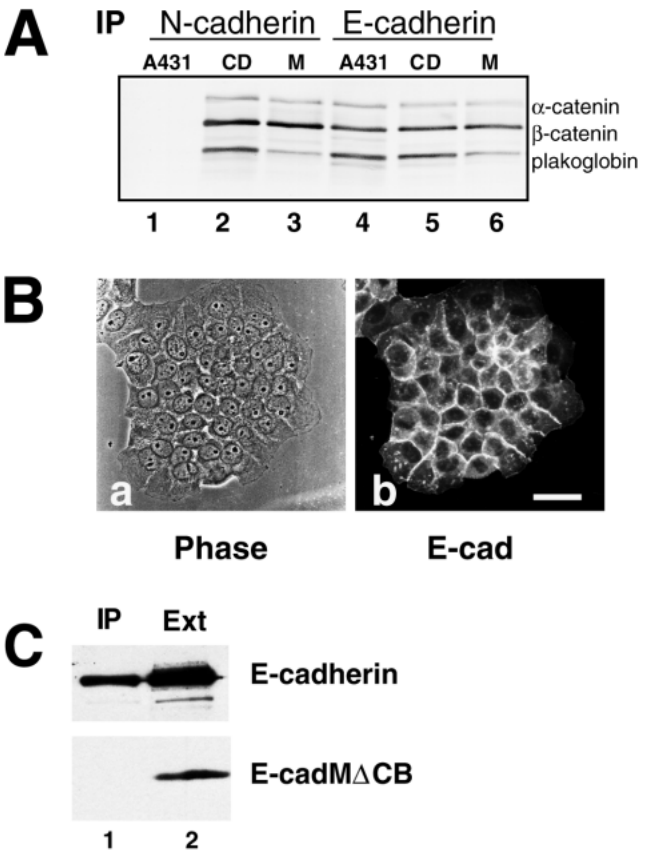


Fig. 5. (A) Catenins associate with truncated cadherins. Extracts of A431, A431-N-cad-CD (CD) or A431-N-cad-M (M) cells were immunoprecipitated with anti-N-cadherin (13A9) or anti-E-cadherin (HECD1). The immune complexes were separated by SDS-PAGE and immunoblotted with a cocktail of monoclonal antibodies against α-catenin (1G5), β-catenin (6E3) and plakoglobin (15F11). (B) Removal of the catenin-binding site abolishes dominant negative activity. A431 cells transfected with E-cad-MΔCB were processed for phase (a) and immunofluorescence microscopy (b) using antibodies against E-cadherin (HECD-1). Bar, 10 μM. (C) E-cad-MΔCB does not associate with catenins. Extract of A431-E-cad-MΔCB cells (Ext) or anti-β-catenin (6E3) immunoprecipitations of extract from A431-E-cad-MΔCB cells (IP) were resolved by SDS-PAGE and immunoblotted for E-cadherin using the cytoplasmic domain antibody.

soluble cytoplasmic domain of either cadherin. However, these immunoprecipitations are not quantitative and thus, we cannot speculate on a specific role for plakoglobin in the dominant negative effect.

Zhu and Watt (1996) have reported that the catenin binding site is required for dominant negative cadherin activity in keratinocytes. To determine if catenin-binding was necessary to produce the dominant negative phenotype in our system, we constructed E-cad-M with the catenin-binding site removed (E-cad-MΔCB). A431 cells stably transfected with E-cad-MΔCB were indistinguishable from the parental A431 cells when examined by phase microscopy or by immunofluorescence microscopy for the localization and level of expression of E-cadherin (Fig. 5B), indicating that the E-cadMΔCB deletion mutant was incapable of acting as a dominant negative cadherin. Immunoprecipitation of extracts of cells expressing

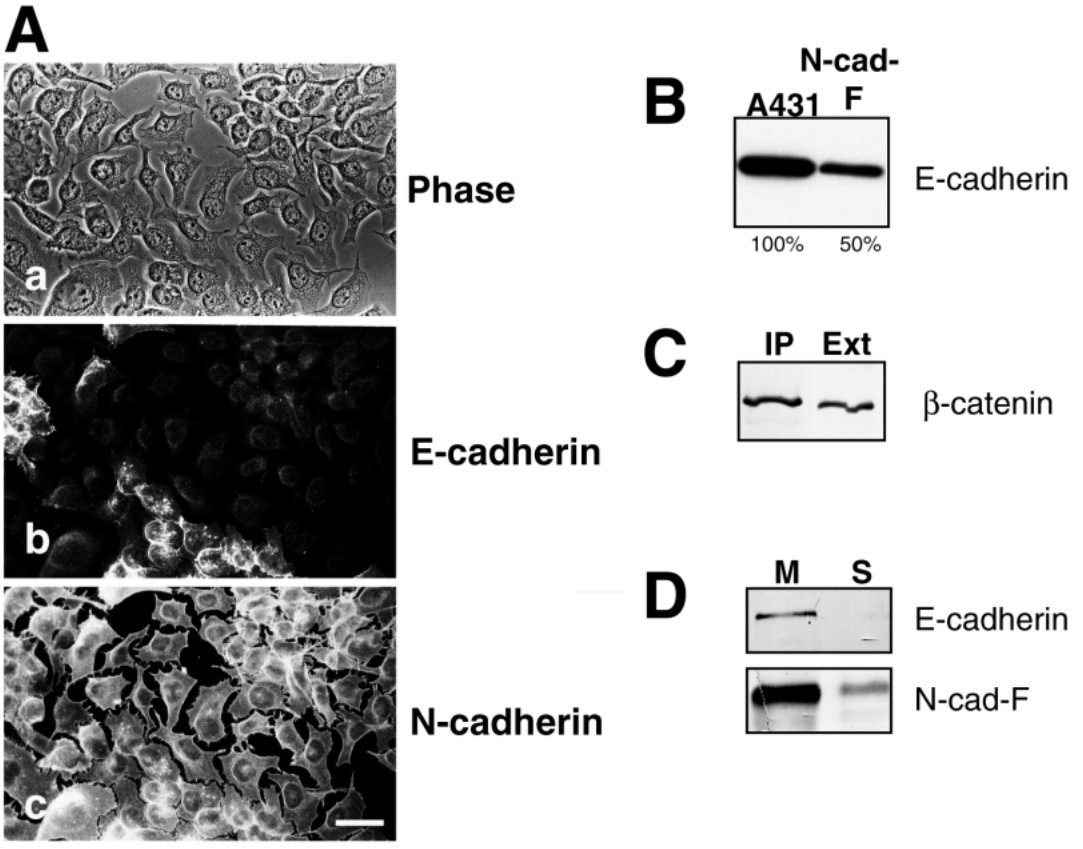


Fig. 6. The orientation of the cadherin cytoplasmic domain at the plasma membrane is not important for the dominant negative phenotype. (A) A431-N-cad-F cells were processed for double immunofluorescence with antibodies against E-cadherin (Jelly; b) and N-cadherin (13A9; c). Note the change in morphology of the cells upon expression of N-cad-F (a). Bar, 10 μM. (B) Confluent monolayers of A431 cells or A431-N-cad-F cells were extracted and equal amounts of protein resolved by SDS-PAGE and immunoblotted with antibodies against E-cadherin (HECD-1). Note the decrease in E-cadherin in N-cad-F cells. (C) Extract of A431-N-cad-F cells (Ext) or anti-N-cadherin (13A9) immunoprecipitations of extracts of A431-N-cad-F cell extract (IP) were resolved by SDS-PAGE and immunoblotted for β-catenin (6E3). (D) Cell extracts were separated into soluble or membrane associated fractions, resolved by 10% SDS-PAGE and immunoblotted with anti-E-cadherin (HECD-1) or anti-N-cadherin (13A9).

E-cad-MΔCB with antibodies against β-catenin showed that E-cad MΔCB did not co-immunoprecipitate with catenins while endogenous E-cadherin did co-immunoprecipitate with catenins, as expected. Thus, we conclude that the cytoplasmic domain of a cadherin must be at the plasma membrane and be associated with catenins in order to behave as a dominant negative cadherin.

The orientation of the cytoplasmic domain at the plasma membrane is not important for the dominant negative phenotype

Recent evidence suggests that lateral clustering of cadherins at the plasma membrane increases the strength of cadherin mediated adhesion and that this clustering requires the juxtamembrane domain (Yap et al., 1998; Ozawa and Kemler, 1998). To examine the possibility that the dominant negative cadherin acts by interfering with lateral clustering of endogenous cadherins, we prepared a molecule in which the cytoplasmic domain of N-cadherin was targeted to the plasma membrane in the reverse orientation. To accomplish this we made use of the farnesylation sequence from Ras which targets the carboxyl terminus rather than the amino terminus to the plasma membrane. Farnesylation requires the C-terminal sequence CaaS, **C**aa**M**, CaaA or CaaG (Clarke, 1992). The complete sequence used in this study was KLNPPDQSGPG<u>C</u>MS<u>C</u>K**C**VL**M** (see Fig. 1C). Following the addition of the farnesyl group, the three most C-terminal amino acids are removed and the resulting C-terminal amino acid, cysteine, is methyl esterified. In addition, palmitic acid is added to the cysteines sixth and ninth from the original end (underlined; Clarke, 1992; Hancock et al., 1990).

N-cad-F was stably transfected into A431 cells and clones were chosen by immunoblot analysis for their expression of N-cad-F protein (not shown). Phase contrast microscopy showed that A431-N-cad-F cells tended to grow as single cells (Fig. 6A, a) and immunofluorescence light microscopy showed that they had high levels of N-cad-F (Fig. 6A, c) with reduced and disorganized E-cadherin (Fig. 6A, b), similar to cells expressing N-cad-M (Fig. 2). Quantitative immunoblot analysis confirmed that A431-N-cad-F cells expressed a reduced level of E-cadherin (Fig. 6B) compared to parental cells. The cells expressing N-cad-F do not appear to have as severe a phenotype as those expressing N-cad-M. That is, although they have decreased cell-cell interactions, they are not

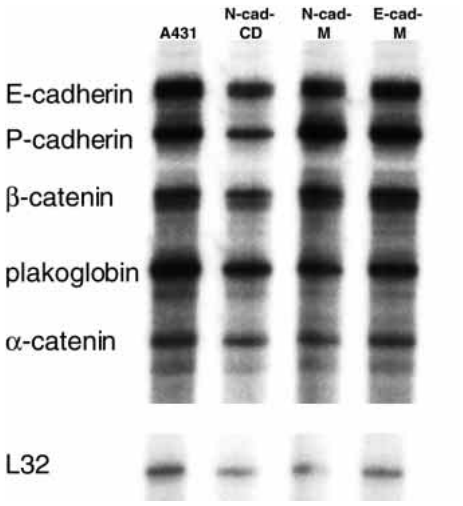


Fig. 7. Cadherin and catenin mRNA levels are unchanged in transfected cells. The level of E-cadherin, P-cadherin, β-catenin, plakoglobin and α-catenin mRNA was compared in A431, A431-N-cad-CD (N-cad-CD), A431-N-cad-M (N-cad-M) and A431-E-cad-M (E-cad-M) cells using RNAse protection. The ribosomal protein L32 was included in the probe set to ensure equal RNA levels in each sample. An antisense probe set was radiolabeled and hybridized with 10 μg total RNA. Single stranded mRNA and excess probe were digested and the protected probes resolved on a 5% acrylamide/8 M urea gel and autoradiographed at –70°C. The length of the protected probes are 360 nt (E-cadherin), 320 nt (P-cadherin), 255 nt (β-catenin), 225 nt (plakoglobin), 202 nt (α-catenin) and 76 nt (L-32).

as rounded as the cells expressing N-cad-M. The ability of N-cad-F to associate with β-catenin was confirmed by immunoprecipitating A431-N-cad-F cell extract with antibodies against N-cadherin and blotting back with antibodies against β-catenin (Fig. 6C). Membrane localization of N-cad-F was confirmed by fractionating A431-N-cad-F cells into membrane associated and cytoplasmic fractions (Fig. 6D). Since N-cad-F is at the plasma membrane in the opposite orientation as the endogenous cadherin, it is unlikely that the juxtamembrane region of N-cad-F is in a position to interfere with lateral clustering of E-cadherin.

Dominant negative cadherins post-transcriptionally down-regulate the levels of endogenous cadherin

To address the mechanism of down regulation of endogenous E-cadherin, mRNA levels of adherens junction components were examined by RNAse protection. We employed an RNAse protection protocol (Hobbs et al., 1993) that allowed us to examine the expression of E-cadherin, P-cadherin, α-catenin, β-catenin and plakoglobin in one reaction. The ribosomal protein L32 was included in the probe set to normalize the data. The level of mRNA for each of the adherens junction components was unchanged in A431-N-cad-CD cells, A431-N-cad-M cells and A431-E-cad-M cells compared to parental A431 cells (Fig. 7). Thus, the dominant negative cadherin does not down regulate endogenous E-cadherin at the level of transcription.

Since the dominant negative cadherin expressing cells have reduced endogenous E-cadherin protein, but do not have reduced levels of E-cadherin mRNA, we examined the stability of endogenous E-cadherin protein. A431 cells, A431-N-cad-M cells and A431-E-cad-M cells were metabolically labeled with [^{35}S]methionine/cysteine for 30 minutes and chased for 0, 4, 8, 12, or 16 hours with non-labeled methionine/cysteine-containing culture medium. Labeled cell extracts were immunoprecipitated with anti-E-cadherin antibodies (HECD1), resolved on 7% SDS-PAGE and autoradiographed at –70°C (Fig. 8). In A431 cells, approximately 40% of the newly synthesized E-cadherin remained after a 16 hour chase. However in A431-N-cad-M cells and A431-E-cad-M cells, only about 10% of the newly synthesized E-cadherin remained in the sample at this time point. Similar results were obtained in three separate experiments; the gels shown are representative examples. Thus, the expression of a dominant negative cadherin leads to increased turnover of endogenous cadherins. In some tumor cells in culture, E-cadherin turnover may be due

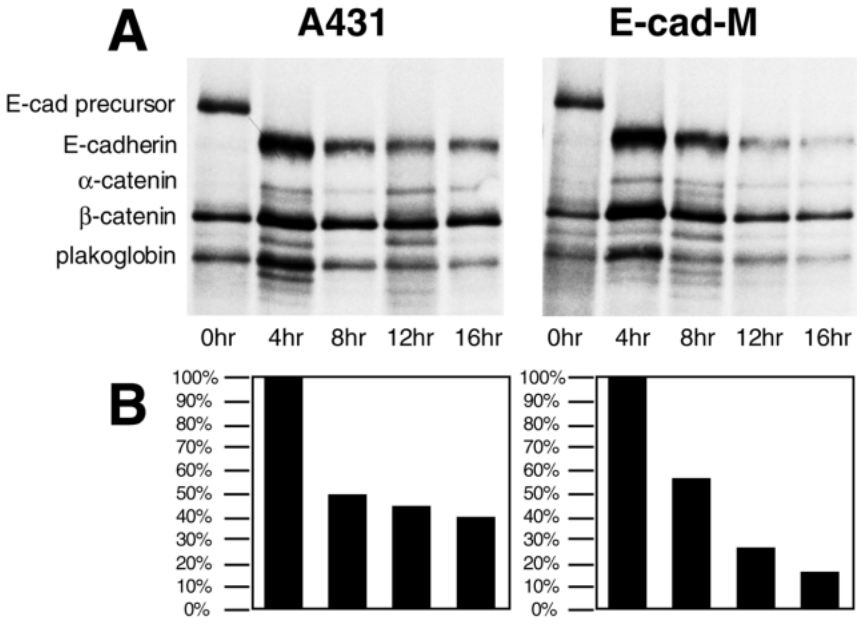


Fig. 8. Transfection of A431 cells with a dominant negative cadherin decreases the stability of endogenous E-cadherin. A431 cells and A431-E-cad-M (E-cad-M) cells were metabolically labeled for 30 minutes and chased for 0, 4, 8, 12 or 16 hours. Extracts of labeled cells were immunoprecipitated with anti-E-cadherin (HECD1) and the immune complexes resolved on 7% SDS-PAGE and visualized by autoradiography. After a 16 hour chase, approximately 40% of E-cadherin was stable in the parental A431 cells compared to 10% in the transfected cells. It should be noted that these immunoprecipitation reactions were washed with stringent RIPA buffer which removes most of the α-catenin.

to release of the extracellular domain of the cadherin as an 80 kDa soluble fragment (Wheelock et al., 1987). To determine if dominant-negative cadherins increased the levels of the 80 kDa E-cadherin extracellular fragment in the culture medium, we collected medium and immunoprecipitated with antibodies against E-cadherin. We did not detect an increase in the levels of the 80 kDa fragment (data not shown) suggesting that release from the cell surface may not be the mechanism of turnover in this case.

DISCUSSION

Three adhesion defective, dominant negative cadherin mutants with truncated extracellular domains and full length cytoplasmic domains have been described. A *Xenopus* dominant-negative N-cadherin (N-cadΔE) disrupted the formation of the ectoderm when injected into the animal pole of a fertilized *Xenopus* egg (Kintner, 1992) and interfered with establishment of cell polarity when expressed in mouse intestinal epithelial cells (Hermiston and Gordon, 1995a). A chicken dominant-negative N-cadherin (cN390Δ) disrupted cell-cell adhesion in a mouse keratinocyte cell line (Fujimori and Takeichi, 1993), and a mouse dominant-negative E-cadherin/H-2Kd fusion protein interfered with terminal differentiation in keratinocytes (Zhu and Watt, 1996). Although the effect of the dominant-negative cadherin was dramatic in each case, the mechanism of its action is poorly understood. This study was designed to elucidate the mechanism of action of such dominant-negative cadherins.

Here, we show that cadherin cytoplasmic domain dominant-negative peptides must be membrane-associated to be active, but the orientation at the cytoplasmic face of the plasma membrane is not important. In addition, to produce the dominant-negative effect in epithelial cells, the truncated cadherin must be able to bind catenins; however, binding catenins, in the absence of membrane association, is not sufficient for activity. Our results suggest that the dominant-negative function is due to decreased expression of endogenous cadherin resulting from increased protein turnover, rather than decreased transcription of the gene.

Full-length functional N-cadherin has been shown to alter the morphology of epithelial cells when expressed ectopically in *Xenopus* embryos (Kintner, 1992) and also in squamous cell carcinoma cell lines (Islam et al., 1996). When fertilized *Xenopus* eggs were injected with N-cadherin mRNA, the cells of the ectoderm had a spindle shaped morphology and did not have tight cell-cell contacts. A similar effect was seen in squamous carcinoma cells, which normally express only E- and P-cadherin, where the expression of N-cadherin correlated with a fibroblastic morphology and loss of cell-cell junctions (Islam et al., 1996). These are similar characteristics to those conferred on epithelial cells expressing a dominant-negative N-cadherin. Thus, one might speculate that the effects may be due specifically to properties in the cytoplasmic domain of N-cadherin. However, we present evidence here that this is not the case. Transfecting either dominant-negative N-cadherin or dominant-negative E-cadherin into A431 cells produces identical cellular phenotypes arguing that they share a common feature. The dominant negative E-cadherin described by Zhu and Watt (1996) further supports this hypothesis.

One possible explanation for the dominant-negative phenotype resulting from exogenous expression of either full length or mutant cadherins is that the cells decrease the level of expression of endogenous active cadherin. This is substantiated by the experiments presented in this study showing that transfecting a fully functional, tagged E-cadherin into A431 cells also resulted in down-regulation of the endogenous cadherin. Thus, the cells apparently regulate the level of expression of total cadherin (at the membrane and bound to catenins), regardless of whether or not it is active as an adhesion molecule. It is somewhat surprising that a 25-50% decrease in endogenous cadherin is sufficient to induce such a dramatic change in cellular morphology, pointing out the importance of regulating precise levels of specific cell surface proteins.

An interesting question to consider is how the cell regulates the level of total cadherin it expresses. This regulation does not appear to involve the adhesive function of the cadherin molecule as both truncated and full length cadherins are effective in down-regulating endogenous cadherin levels. Down-regulation of endogenous cadherin requires that the dominant negative cadherin be at the membrane and also be capable of binding catenins. A non-membrane targeted cadherin cytoplasmic domain that binds catenins is inactive as a dominant negative cadherin, suggesting that β-catenin signaling most likely is not responsible for regulating the levels of cadherins expressed by a cell. To further support this, we saw no evidence for nuclear accumulation of β-catenin or the β-catenin/cadherin complex in cells transfected with any of the truncated cadherin constructs (data not shown). Thus, it may be that association of the cadherin/catenin complex at the plasma membrane with the actin cytoskeleton is important for informing the cell of its cadherin levels.

One caveat to our studies is that the dominant-negative cadherins we used were targeted to the membrane with lipid tags, not with a transmembrane domain, which makes them different from any reported dominant-negative cadherin constructs. Initially, these constructs were designed to determine if the transmembrane domain of the cadherin was important for generating the dominant-negative phenotype. We thought the transmembrane domain may be important because it is so highly conserved across species. As it turned out, targeting the cytoplasmic domain to the membrane with a lipid tag produced a very efficient dominant-negative cadherin. Moreover, both the farnysyl-tagged cadherin cytoplasmic domain and the myristoyl-tagged cadherin cytoplasmic domain were active in producing the dominant-negative phenotype, illustrating that the orientation within the membrane is not crucial for this activity.

Recently the juxtamembrane region of cadherins has been implicated in lateral dimerization (and clustering) of cadherins at the plasma membrane and binding of $p120^{ctn}$ (Yap et al, 1998; Ozawa and Kemler 1998). One possible explanation for the scattered phenotype of cells expressing dominant-negative cadherin constructs is that the functional endogenous cadherin cannot cluster within the plane of the membrane and thus cannot form stable junctional complexes. Our data suggest that this may not be the case because the N-cadherin cytoplasmic domain targeted to the membrane with a farnysyl tag is effective at producing the dominant negative effect, even though it is present with the C terminus at the membrane rather

than the N terminus. It seems unlikely that this peptide is positioned in a way such that it could interfere with clustering of cadherins in the membrane. However, since the three dimensional structure of the cadherin cytoplasmic domain is not known, it is possible that the cytoplasmic domain folds in such a way that would allow N-cad-F to interfere with clustering. Further support for the idea that the dominant negative cadherin is not acting solely by interfering with lateral clustering of the endogenous cadherin is evidenced by the observation that the E-cadherin cytoplasmic domain missing the catenin binding site (E-cad-MΔCB) does not produce the dominant-negative phenotype when expressed in epithelial cells. The juxtamembrane region of E-cad-MΔCB is intact and in the same position as that of E-cad-M which has the catenin binding site and does produce the dominant negative phenotype when expressed in epithelial cells. This brings us back to the idea that it is the decrease in endogenous cadherin that is responsible for the scattered phenotype of epithelial cells expressing a dominant-negative cadherin. This idea is supported by the fact that expression of intact functional N-cadherin in epithelial cells produces morphological changes similar to those observed when a dominant negative cadherin is expressed by these cells and also results in down-regulation of E-cadherin expression. It is not clear why the exogenous functional N-cadherin does not promote the typical epithelial cell morphology, but we surmise from these data that N-cadherin and E-cadherin, in the context of the epithelial cell, do not promote the same kind of cell-cell interactions. One might propose that N-cadherin interferes with lateral clustering of E-cadherin in an epithelial cell expressing both cadherins. However, squamous epithelial cells express both E-cadherin and P-cadherin and we have shown that these two cadherins are not found in the same complexes and thus must be able to sort out from one another (Johnson et al., 1993). One might expect E-cadherin and N-cadherin to do the same thing when expressed in the same cell.

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